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HUMAN MONOCLONAL ANTIBODIES AND METHODS FOR HUMAN MONOCLONAL ANTIBODY PRODUCTION

Background of the Invention

Immunotherapy is a leading candidate for the successful treatment of a variety of conditions including cancer and allergy. Immunodiagnostics and tracing agents that are administered to human beings also are desirable. The need for human monoclonal antibodies which do not provoke an immune response when administered to a human is clear. The production of human monoclonal antibodies, however, is problematic. Firstly, in vivo immunization of human beings presents important ethical and medical considerations, and therefore limits the number of antigens that can be used for provocation of a human immune response. Secondly, in vitro attempts to produce human monoclonal antibodies have been severely limited by the inability to generate both a primary and secondary immune response in vitro, and the production of monoclonal antibodies by the known methods have been largely unsuccessful in producing high-affinity antibodies.

Human monoclonal antibodies have been produced using two general methods: (1) direct immortalization of antibody-producing human lymphocytes with Epstein-Barr Virus (EBV) and (2) hybridoma formation between immortalized human or murine cell lines and human lymphocytes from an immunized host.

Although the most readily available source of human lymphocytes is peripheral blood, peripheral

blood lymphocytes (PBL's) have consistently performed very poorly when used for the production of human monoclonal antibodies, and peripheral blood has been considered to be a suboptimal source of lymphoid cells for this purpose. See C. Borrebaeck, J. Immunol. Methods, 123: 157-165 (1989) and references cited therein. In particular, workers have speculated that the antibody-producing human B cells found in peripheral blood may be less amenable to formation of lymphoblast cells as compared to B cells in other lymphoid compartments such as the spleen and lymph nodes. There is a generally unfavorable ratio of T suppressor cells to B cells in the peripheral blood of humans. Moreover, it has been speculated that peripheral blood does not contain enough antigen-presenting cells to permit in vitro immunization.

Various complex and time consuming procedures have been developed to activate peripheral blood lymphocytes during in vitro immunization. Some investigators have attempted to separate individual blood cell populations and reconstruct a mixture of T, B and accessory cells at a fixed ratio in order to promote in vitro antigen-specific activation of peripheral blood lymphocytes. See L. Danielsson et al., Immunology, 61:51 (1987) and M.-K. Ho, "Production of monoclonal antibodies by in vitro immunization", Immunology Series; Vol. 30, M. Dekker, N.Y. (1987). In other attempts at in vitro immunization, suppressor cells have been depleted by

physical separation, lysis with antibodies or reaction with various chemicals. See K. James and G.T. Bell, J. Immunol. Methods, 100:5-40 (1987) and references cited therein.

Whatever the exact explanation for the poor performance of PBL's, the lack of a well-established technique for in vitro immunization of human blood cells remains a significant obstacle to human monoclonal antibody development.

Summary of the Invention

This invention pertains to cells and cell lines derived therefrom that are capable of secreting a high-affinity human monoclonal antibody specific against a selected antigen. The invention also pertains to in vitro immunization methods of making these cell lines and to therapeutic and diagnostic constructs utilizing these monoclonal antibodies.

The invention provides a method for making a human monoclonal antibody that is specific against a selected antigen. To achieve this, a mixed lymphocyte reaction mixture is formed. This mixed lymphocyte reaction mixture includes a preparation of human lymphocytes, the preparation enriched for memory B-lymphocytes and obtained from a donor that is seropositive for the selected antigen, a preparation of human lymphocytes from a second donor that is allogeneic with respect to the first donor, and the selected antigen. The preparations and the antigen are present in sufficient quantities and subjected to

conditions whereby the mixed lymphocyte reaction is generated between lymphocytes of the two preparations, characterized by the production of secondary lymphoblast progeny of the memory B-lymphocytes. These secondary lymphoblasts then are fused with heterohybrid fusion partners, and a fused product that secretes antibody with high affinity binding specificity for the selected antigen is isolated.

In preferred embodiments, antibodies with specificity for human self-antigens such as immunoglobulins, antigens involved in autoimmune disease and cancer antigens are obtained. Antibodies with specificity for foreign antigens such as bacterial, viral, parasitic, and other infectous agents also are obtained. Preferably, the preparation of lymphocytes from the second donor is a preparation of splenocytes, and preferably the memory B-lymphocytes are obtained from peripheral blood of the first donor.

In another aspect of the invention, the memory B-lymphocytes are obtained from a nonhuman mammal having a human immune system. This permits the preparation of particular antibodies resulting from the immunization of the nonhuman mammal, thereby avoiding ethical and medical considerations. In the preferred process, the nonhuman mammal is a SCID Mouse that is reconstituted with human memory B cells and immunized with the selected antigen. Memory B cells then are collected from the SCID Mouse.

Detailed Description of the Drawings

Figure 1 summarizes results of an enzyme immunoassay procedure for identifying donors (x axis) having serum containing anti-IgE antibodies.

Figure 2 presents assay results of fusion product screening for those cell lines producing human IgG anti-human IgE monoclonal antibody (wells 1-24).

Figure 3 presents assay results of fusion product screening for those cell lines producing human IgG anti-human IgE monoclonal antibody (wells 25-41).

Detailed Description of the Invention

A. The Immune Response

Antibodies of the present invention are made in an *in vitro* immunization procedure in which memory B-lymphocytes from a seropositive donor are activated to form secondary lymphoblasts in a secondary immune response. Secondary lymphoblasts then are fused to an immortal fusion partner cells to form a cellular fusion product.

Immunity in vertebrates is effected by antigen-binding antibody molecules that are secreted by cells of the B-lymphocyte lineage. The differentiation of B-lineage cells is believed to be divided into two general stages. The first stage is antigen-independent and is involved in differentiation of stem cells to B lymphocytes. These B-lymphocytes are defined by the presence of surface immunoglobulin that functions as an antigen receptor. In mammals,

this process occurs in the fetal liver and subsequently is maintained continuously in the bone marrow of adults. Each newly generated B-lymphocyte cell and its progeny that have never been exposed to antigen expresses a novel species of immunoglobulin molecule on its surface with a unique set of binding specificities. After expressing this surface immunoglobulin, B-lymphocytes migrate to peripheral lymphoid organs such as the spleen and lymph nodes where contact can occur between these "naive" lymphocytes and circulating antigens. In the absence of any contact with antigen, these B-lymphocytes are non-dividing.

The second stage of B-lymphocyte differentiation is antigen dependent and involves specific contact and binding of antigen to the surface immunoglobulin of a "naive", non-dividing B-lymphocyte in the peripheral lymphoid organs of the spleen and/or lymph nodes. This process, in combination with a complex series of interactions between T-lymphocytes, macrophages and soluble growth factors called "cytokines", forces that particular cell to proliferate in the spleen and/or lymph nodes into a proliferating clone of primary "lymphoblast" cells. The cells in the clone then differentiate into non-dividing cells called "primary plasma cells" and/or memory B-lymphocytes. Primary plasma cells are the effector cells of the immune system in the spleen, lymph nodes or peripheral circulation which secrete into the bloodstream large amounts of an immunoglobulin molecule, primarily of

class IgM, although IgG, IgA and IgE may also be elicited if the naive B-lymphocytes are exposed to various soluble factors. The immunogloblulin molecule has the same binding specificity as that present on the membrane of the naive B-lymphocyte. This process is called clonal selection or the "primary immune response" and the individual is now defined as being "seropositive". It will be readily appreciated by those of ordinary skill in the art that an individual may be exposed to a selected antigen, in an amount sufficient to lead to memory B-cell formation, but that individual no longer expresses detectable levels of antibody specific for that selected antigen in the serum. Thus, the term "seropositive" also is meant to encompass individuals having memory B-cells, but undetectable serum levels of antigen-specific antibody.

The "memory B cell" generation requires proliferation of B lymphocytes and clonal selection by identical antigen (stimulated by cytokines). These cells do not secret antibody. Clonal proliferation of B-lymphocytes destined to become memory cells occurs in well-defined locations called "germinal centers" in the spleen and lymph nodes.

After some time, the memory cells leave the lymph organs and enter peripheral circulation. Non-memory or "naive" B-lymphocytes do not recirculate.

The memory B-lymphocytes are major components of peripheral blood lymphocytes in seropositive individuals. They are ready to respond in an

accelerated manner, relative to naive lymphocytes, to further contact with the same antigen that produced the primary response by proliferating into so-called "secondary lymphoblasts". The proliferating secondary lymphoblasts and their progeny differentiate into secondary plasma cells. This response of memory B-lymphocytes to the same antigen is called the "secondary immune response".

Significantly, memory cell formation produces new sets of antigen binding sites to deal more efficiently with that antigen on a second encounter. These new binding sites are believed to be generated through somatic mutation and selection in so-called "affinity maturation." In this memory cell pathway, a population of B-lymphocytes is generated with drastically changed antibody binding regions from those B-lymphocytes developed in the primary immune response. From this process, secondary plasma cells express much higher affinity antibodies in the secondary immune response than do the primary plasma cells of the primary immune response. Moreover, primarily IgG is produced, rather than IgM as in the "primary" response.

Memory B-lymphocytes are not easy to distinguish morphologically from "naive" B-lymphocytes. Nevertheless, there are a number of non-Ig surface markers that are differentially expressed on naive and memory B-lymphocytes. These include, peanut-agglutinin-binding molecules (PNA), lymphocyte adhesion molecule MEL-14, and antigen J11d. See

Vitteta et al., Ann. Rev. Immunol. (1991). Table I, modified from Vitteta et al., summarizes this comparison.

Table I. Comparison of Naive and Memory B-lymphocytes

Characteristic	Naive	Memory
Surface markers		
Ig isotype	IgM/IgD	IgG/IgE/IgA
J11d	High	Low
MEL-14	Low	High
Complement receptor	Low	High
PNA molecules	Low	High
Recirculation	No	Yes
Ig-variable regions	Nonmutated	Mutated
Ag affinity	Low	High

This invention takes advantage of the fact that high-affinity antibodies are generated from secondary lymphobast progeny, and involves formation of immortalized monoclonal antibody producing cell-lines preferentially from such secondary lymphoblasts.

As used herein, "fusion product" refers to stable, immortalized antibody producers which result from the fusion of a human antibody-secreting peripheral blood lymphocyte with a human chromosome-containing heterohybrid (fusion partner). The terms "heterohybrid" or "fusion partner" are used

interchangeably and refer to an immortal cell line which retains detectable human-derived chromosomes and does not secrete immunoglobulin.

"Cell line" refers to various embodiments including, but not limited to, individual cells, harvested cells and cultures containing the cells so long as these are derived from cells of the cell line referred to. By "derived" is meant progeny or issue. It is known in the art that spontaneous or induced changes can take place in human chromosomes during storage or transfer. Therefore, cells derived from the cell lines referred to may not be precisely identical to the ancestral cells or cultures, and any cell line referred to include such variants.

The term "high affinity" refers to monoclonal antibodies with binding affinities greater than 10^8/mol; preferably greater than 10^9-10^{10}/mol.

B. General Method

A human chromosome-containing "fusion partner" is prepared by fusing mouse and human (mouse x human) or human and human (human x human) cell lines together, at least one of the pair of cells being a neoplastic lymphoid cell such as a myeloma or lymphoma.

A preparation of cells prepared by forming a population enriched with secondary lymphoblast cells also is prepared. The preparation of secondary lymphoblast cells may be derived from a population of lymphocytes enriched for memory B-lymphocytes of a seropositive human donor. By "enriched" it is meant a

preparation containing a relatively high proportion of memory B-lymphocytes such as is present in PBL's circulating in blood or located in tonsils or lymph nodes. Enriched populations also may be found in the germinal centers of the spleen (and collected, for example, by micropipetting). An "enriched" population is to be contrasted with that of the spleen, which generally contains a relatively low ratio of memory B lymphocytes to other cells.

The human-chromosome containing fusion partner is fused with the secondary lymphoblast cells and clones of the fusion product that secrete a particular human monoclonal antibody against a particular antigen are selected.

The preparation of cells enriched for secondary lymphoblasts is provided by generating a mixed lymphocyte response between the enriched memory cell preparation of the seropositive donor and lymphocytes from an allogeneic donor. The term "mixed lymphocyte response" refers to stimulation of cell proliferation by growth factors, and other proliferative agents, formed during co-culture of lymphocytes from allogeneic donors.

Fusions are carried out using standard conditions known in the art, such as incubation in the presence of polyethylene glycol (PEG), followed by selection for the desired fusion products.

1. Identification of Suitable Donors:

Individuals with serum antibody directed against

the antigen of interest are identified by first screening the suspected individual's serum using any appropriate assay such as an enzyme-linked immunoassay (ELISA)®, an enzyme immunoassay (EIA), a hemagglutination assay (HA), or a complement fixation assay (CF). These serum screening methods are well-known to those of ordinary skill in the art.

Another method of identifying these individuals is for an initial study to be performed of a suspected donor population to select individuals with a specific disease, an autoimmune condition, a specific immunization, or other predisposing factors for specific antibody production. Particular individuals of this type are discussed in more detail below in §C2.

Individuals who either are selected on the basis of their known immunological history or are screened using appropriate assay proceed to the next phase of the general method.

2. Screening for Active PBL's:

Individuals are screened to determine if they have peripheral blood lymphocytes actively secreting antibody to the selected antigen. A particularly preferred screening assay is an ELISPOT (enzyme-linked immunospot) assay as generally described by C.C. Czerkinsky et al., J. Immunol. Methods 65: 109-121 (1983) and B.D. Mazer et al., J. Allergy Clin. Immunol. 88: 235-243 (1991). Generally, donors with greater than 50 ELISPOT-positive cells per million peripheral blood lymphocytes screened are considered

to be positive. Those peripheral blood lymphocytes are used in the next immunization and activation step.

3. Immunization and Activation of PBL's:

A significant aspect of the method of the invention is that the memory B-lymphocytes of a population of PBL's are activated in vitro to form secondary lymphoblasts by co-culturing the lymphocytes in the presence of the selected antigen and lymphocytes from an allogeneic donor.

The ratio of PBL's to allogeneic lymphocytes is about 1:1, although this ratio can be adjusted easily to determine an optimum ratio. The concentration of selected antigen can be varied over a wide range, preferably between about 0.05 and about 10 micrograms/ml of culture. Under circumstances where the selected antigen is a tumor cell marker, the tumor antigen may be in the form of intact tumor cells, thus the actual amount of antigen present may be difficult to measure. In these situations, sufficient antigen to cause secondary lymphoblast formation in a secondary immune response is determined visually by microscopic observations of proliferations and cell growth.

The co-culturing of PBL's and allogeneic lymphocytes occurs for a 3-5 day period, although 4 days is considered optimal. Viable lymphocytes then are recovered for the next fusion step.

4. Preparation of Fusion Product:

The fusions to form the fusion products of the present invention are performed basically by the method of Kohler and Milstein, Nature, 256: 495 (1975), the entire contents of which are incorporated herein by reference. Briefly, an immortalized cell line comprising the fusion partner of the present invention is combined with secondary lymphoblasts derived from peripheral blood lymphocytes using a fusogen to destabilize the cell membranes of the lymphoblasts and fusion partner. Fusogens include phosphoglycerides and sterols. Preferably, 40%-50% polyethylene glycol of molecular weight of about 1000 - 4000 at room temperature to about 37°C to 40°C is used. Fusion requires about 5-10 minutes and the cells are then centrifuged, resuspended and seeded in culture.

5. Production, screening and purification of human monoclonal antibodies:

After fusion with human secondary lymphoblasts in accordance with the procedures set forth herein, the immortalized fusion products of the invention are selected. This can be accomplished by using an appropriate selection medium. The preferred secondary lymphoblasts are derived from normal peripheral blood lymphocytes, and an appropriate selection medium can be the HAT or AH medium which will not allow growth of unfused peripheral blood lymphocytes as well as unfused heterohybrids. Alternate selection procedures

are possible depending upon the nature of the cells used in the fusion. It would be possible to confer by mutagenesis alternate sensitivities on the immortalizing fusion product which would respond to other medium selecting factors besides HAT and AH.

Clones having the required specificity for the selected antigen are identified by assaying the culture medium for the presence of antibody with the ability to bind to the desired selected antigen, and the nature of the monoclonal antibodies may be further characterized by testing for affinity and immunoglobulin class (e.g. IgM, IgG, IgA, IgE) using standard methods. Clones that produce human monoclonal antibodies having the desired specificity may be subcloned by limiting dilution techniques and grown in vitro in culture medium or injected into selected host animals and grown in vivo.

The antibodies may be separated from resulting culture medium by conventional antibody fractionation procedures such as ammonium sulfate precipitation, DEAE cellulose chromatography, affinity chromatography and the like.

C. Components

1. Fusion Partners

The fusion partners used in the methods of the present invention are a plurality of cells that are fused. The fused cells are characterized as being immortal and having some portion of their chromosomes from a human and another portion of their chromosomes

from a non-human mammal, particularly a lagomorph or rodent, more particularly, a rodent such as a mouse. Alternately, all the chromosomes of the fusion partner could come from humans. For example, the fusion partner could be a human lymphocyte fused with a human myeloma cell, as exemplified by United States patent 4,529,694 or the fusion partner could be a human lymphocyte fused with a human plasmacytoid cell, as exemplified in U.S. Patent 4,434,230. See also, Larrick, (U.S. Patent 5,001,065) for summary of various fusion partners containing human-chromosome cell lines as well as Kaplan et al., (U.S. Patent 4,574,116) and references cited therein.

Particularly preferred fusion partners for use in the present invention are the mouse x human heterohybrids constructed by Carroll et al., J. Immunol. Methods, 89, 61-72, (1986) the contents of which are incorporated herein by reference. The most preferred heterohybrid of this type is the K6H6/B5 mouse x human heterohybrid (ATCC #CRL 1823) in which the murine fraction is derived from a non-immunoglobulin secreting mouse myeloma and the human fraction is derived from a malignant lymphoid cell of a human patient with nodular lymphoma. The fusion partner cell contains at least one, and preferably several, human chromosomes.

Screening and selection procedures to ensure growth of fusion partners and fusion products are important in methods of the invention. Commonly used fusion partners are incapable of growth on certain

selective media chosen to deprive the cells of their ability to synthesize DNA. Two very commonly used media of this description are hypoxanthine-azaserine-thymidine (HAT) medium and azaserine-thymidine (AH) medium. Both of these media take advantage of the capacity of normal cells (i.e. peripheral blood lymphocytes) to utilize an alternate pathway for DNA synthesis under circumstances where their normal synthesis of DNA is inhibited by HAT or AH media. This alternate process is not available in the preferred fusion partner cell lines because they lack a particular enzyme (HGPRT) needed for the alternate pathway of DNA synthesis. Normal cells can grow in a HAT or AH medium even without their normal pathway of DNA synthesis and fusion products can grow in HAT or AH medium since they acquire the normal cell's ability to make HGPRT. Only fusion products, however, can grow and survive in repeated transfers in HAT or AH medium. Although normal cells (PBL's) can grow in HAT or AH, they cannot survive because they are not immortalized and thus do not survive repeated transfers.

The PBL's can, however, also be immortalized, for example, by transformation with Epstein-Barr virus. These PBL's can then be activated to form secondary lymphoblasts according to the invention. In these circumstances where the fusion partner cell is to be fused to a secondary lymphoblast derived from a peripheral blood lymphocyte immortalized with Epstein Barr virus, an additional selection property is

required. The immortalized PBL's do not die from multiple transfers as would normal blood lymphocytes, and they will grow in HAT or AH medium. Thus, a screening procedure using immortalized PBL's requires inclusion in the medium of a drug (e.g. oubain) to which these immortalized lymphocytes are sensitive. Therefore, the fusion partner must have an acquired resistance to this drug so that it can transfer resistance to the immortalized PBL and allow the resulting fusion product to grow.

2. Lymphocytes from a Seropositive Donor

Peripheral blood lymphocytes from a first, seropositive donor are activated to form secondary lymphoblasts and these secondary lymphoblasts are fused with the fusion partner to form a fusion product. Memory B-lymphocytes having already undergone affinity maturation in a seropositive donor are preferred particularly because of the high affinity antibodies that are produced as the result of the affinity maturation.

The most readily available, and preferred, enriched source of memory B-lymphocytes is the peripheral circulating blood, rather than the tonsils, lymph nodes, lymph ducts or germinal centers in the spleen. Nevertheless, preparations of cells enriched for memory B-lymphocytes may also be obtained from enlarged lymph nodes, tonsils, lymph ducts or germinal centers in the spleen by surgical procedures well-known in the art. For example, lymphocytes

enriched in memory B-lymphocytes can be obtained from the lymph ducts by cannulating the thoracic duct or by peritoneal lavage.

Where production of a human monoclonal antibody specific against a selected antigen associated with an infectious agent such as a parasite, virus or bacterium is desired, one possible source of PBL's are those from seropositive individuals with an infection caused by the identical infectious agent. Therefore, PBL donors can be humans who have developed an immune response to a particular parasite, bacterium or virus, thus leading to enrichment of the peripheral blood with memory B-lymphocytes having the capacity to form secondary lymphoblast cells.

Other donors in this category are those previously exposed to an infectious agent, other nonself-antigen or self-antigen, e.g. HLA, such as during pregnancy or as the result of a transfusion. In particular, transfusions into patients with immunoglobulin deficiencies can provide a source of peripheral blood lymphocytes that are particularly important in generating antibodies according to the method of the invention. For example, IgA deficiency is a common immunological disorder that is sometimes associated with an immunodeficiency syndrome, allergic disease, and/or autoimmune disease. Many subjects with this deficiency are healthy even with the IgA deficiency. The IgA deficiency is but one manifestation of a defect in B-lymphocyte differentiation that could also result in IgE deficiency, IgG2 and/or IgG4 deficiency. See M.A.H.

French and R.L. Dawkins, Immunol. Today, 11:271-273 (1990) and references cited therein. IgA deficient individuals will generate B-lymphocytes producing anti-IgA antibody after receiving blood products containing serum IgA. Therefore IgA (or IgE) -deficient individuals that have been transfused with blood products containing serum IgA (or IgE) can be a particularly preferred source of peripheral blood lymphocytes for use in methods of the present invention that produce monoclonal human anti-human IgA (or IgE) antibody.

Generally speaking, deliberate inoculation of humans with a selected antigen is also possible, provided that the substance to be injected is available in adequate amounts, is non-toxic and capable of initiating an immune response. Although the range of antigens which can be injected, together with the appropriate immunization schedules, may be limited by ethical and other considerations, seropositive donors having acceptable peripheral blood lymphocytes can be obtained by immunizing individuals following approved schedules using bacterial, viral, or other antigens. For example, deliberate inoculation of humans with bacterial lipololysaccaride (LPS) has been performed. See J.D. Baumgartner et al., J. Infectious Diseases, 163: 769-772 (1991).

In other embodiments of the invention, the peripheral blood lymphocytes are derived from a seropositive donor, whose seropositive nature is caused by an autoimmune disease. Such individuals

typically have lymphocytes with specificity for a "self" antigen.

In further embodiments, the peripheral blood lymphocytes are derived from a seropositive donor, whose seropositive nature is caused by a disease state, such as ovarian cancer. The antigen CA-125 is a cell marker for these cancers. The term "self" antigen is meant to encompass not only those antigens characteristic of a healthy person but also those characteristic of certain unhealthy or abnormal states, e.g., cell markers for cancer cells as well as the large number of cell determinant (CD) markers known to those of ordinary skill in the art.

A non-human source of PBL's may also be used after deliberate inoculation with antigen to produce infection. A particularly preferred non-human animal for use in the methods of the present invention is the SCID mouse, (M.J. Bosma et al., "The SCID Mouse", Current Topics in Microbiol. and Immunol., 152: Springer-Verlag, 1989). The SCID (severe combined immune deficiency) mouse is characterized by the absence of both B- and T-lymphocyte immunity. The peripheral blood of these mice shows a lack of lymphocytes. Lymphoid tissues are 1/10 or less normal size and spleen and lymph node areas are severely deficient in lymphocytes as well. Functional human immune systems have been successfully transferred to, and reconstituted within, these SCID mice. D.E. Mosier et al., Nature, 335: 256-259 (1988), incorporated herein by reference. Human bone marrow injected into a SCID

mouse will generate human lymphocytes that can be immunized. Generally, non-human origins for PBL's are not desirable for producing human monoclonal antibodies for therapeutic applications, since the resulting antibodies retain their non-human character and may induce anti-antibody formation. Nevertheless, the SCID mouse, with a transplanted human immune system, or other non-human hosts having similar characteristics, are suitable PBL donors for methods of the invention.

While the monoclonal antibodies that are specifically exemplified herein are human IgG, the invention is not limited to any particular class or subclass of human monoclonal antibody. Thus, specific monoclonal antibodies may be IgG1, IgG2, IgA, IgE and IgM.

3. Allogeneic Lymphocytes

A significant feature of the present invention is the co-culture of allogeneic lymphocytes with the peripheral blood lymphocytes from a seropositive, first donor to provide conditions needed for secondary lymphoblast proliferation. Lymphocytes from a first donor will interact with lymphocytes from a second, genetically dissimilar donor to generate the well-known "mixed lymphocyte response". Briefly, the mixed lymphocyte response is generated because of a mismatch in Major Histocompatibility Complex (MHC) proteins between the two donors.

Each vertebrate species has an MHC identified

originally through its ability to evoke very powerful transplantation rejections. Each MHC contains three clusters of genes. Class I genes encode MHC Class I peptides associated with the cell surface, Class II genes encode Class II molecules that are transmembrane heterodimers and Class III genes encode Class III components linked to the formation of convertases. The genes in a given MHC gene cluster are usually inherited as a single Mendelian trait. Class I molecules are present on virtually all cells of the body; Class II molecules are particularly associated with B lymphocytes and macrophages.

Once lymphocytes from allogeneic individuals (i.e. genetically dissimilar individuals from the same species as the first donor and having different MHC gene clusters) are cultured together in the presence of a selected antigen, the lymphocytes from one individual will recognize primarily the Class II molecules on the cells from the second individual and will proliferate into secondary lymphoblast cells. Helper T-lymphocytes also will develop.

The nature of B-lymphocyte proliferation in this process has not been entirely elucidated. Without wishing to be bound by any theory, however, it is believed that B-lymphocytes of the first donor are activated in the presence of a selected antigen in the mixed lymphocyte response either by: (i) non-specific activation, (ii) reactions associated with signals from macrophages, and/or (iii) signals from helper T lymphocytes recognizing the selected antigen in

association with Class II MHC. The T-lymphocytes produce a series of soluble growth factors (cytokines) which expand antigen activated B-lymphocyte populations causing proliferation into secondary lymphoblasts and somatic mutation with potential for affinity maturation. Other cells, such as macrophages, present in the mixture of lymphocytes, also produce soluble growth factors. Growth factors can include, but are not limited to, the various interleukins and interferons.

The lymphocytes needed to generate the mixed lymphocyte response can be from any allogeneic donor. Lymphocytes capable of generating a mixed lymphocyte reaction can be derived from PBL's, lymph glands, or tonsils of an allogeneic donor. Preferred cells are allogeneic splenocytes. The ability of these lymphocytes to generate a mixed lymphocyte response can be easily determined prior to actual production of monoclonal antibodies by co-culturing the two sets of lymphocytes and measuring the degree of proliferation of the first donor's lymphocytes using incorporation of radioactive thymidine into their DNA, a technique well-known to those of ordinary skill in the art.

A particular and significant advantage of utilizing the mixed lymphocyte response in methods of the present invention is that this procedure avoids the time consuming and complex methods of the prior art for providing growth factors for peripheral blood lymphocytes to stimulate lymphoblast formation. Thus, methods of the present invention avoid freeing

peripheral blood lymphocytes of T cells or T suppressor cells; separating various components of peripheral blood lymphocytes on columns, specifically lysing suppressor cells with monoclonal antibodies; separating lymphocytes into various fractions and reconstituting them; and/or inactivating toxic T cells. Another advantage is the surprising level of successfully producing the desired human, monoclonal-antibody producing cell lines.

4. Antigen

The antigen used to generate the secondary immune response in methods of the invention can be substantially identical to the antigen that initiated the primary immune response in the first donor. Antigens can be derived from a wide variety of bacterial, viral, or other sources that originate extra-corporeally (i.e. "foreign" antigens). Examples of bacterial foreign antigens can include diphtheria toxoid, Pseudomonas aeruginosa, tetanus toxoid, Haemophilus influenzae and the like.

Viral foreign antigens can be derived from hepatitis A and/or B, HIV-1, influenza, and rubella and the like; parasitic antigens can include those derived from Schistosoma mansoni, Leishmania sp., Trypanosome brucei, Clonorchis sinensis, Plasmodium falciparum and others. See, K. James and G.T. Bell (J. Immunol. Methods, 100: 5-40 (1987) for review of immunization procedures and antigens used for in vitro immunization, the entire contents of which are incorporated herein by reference.

As discussed previously, peripheral blood lymphocytes can be derived from a donor having an autoimmune disease. Therefore, the particular antigen is a self-antigen (i.e. one of the donor's own cellular and/or tissue constituents). It will be appreciated that, because there is a wide range of autoimmune diseases, the particular cellular constituents against which antibodies are made can range from specific molecules such as thyroglobulin (in the case of Hashimoto's thyroiditis) to antigens that are not defined to any one organ, such as DNA (in the case of systemic lupus erythematosus). Examples of autoimmune diseases and their associated self-antigens are included in Table II.

TABLE II. Autoimmune diseases and self-antigens

DISEASE	SELF-ANTIGEN
Hashimoto's thyroiditis	Thyroglobulin
Primary myxoedema	2nd colloid Ag (CA2)
	Thyroid peroxidase: Cytoplasmic
	Cell surface
Thyrotoxicosis	Cell surface TSH receptors
	Growth receptors
Addison's disease	Cytoplasm of adrenal cells
Premature onset of menopause	Cytoplasm of steroid-producing cells
Male infertility (some)	Spermatozoa
Insulin-dependent (juvenile) diabetes	Cytoplasm of islet cells
	Cell surface
Type B insulin resistance c acanthosis nigricans	Insulin receptor
Atopic allergy (some)	ß-Adrenergic receptor
Myasthenia gravis	Skeletal and heart muscle
	Acetylcholine receptor
Eaton-Lambert syndrome	Ca^{2+} channels in nerve endings
(Multiple sclerosis)	Brain
Goodpasture's syndrome	Glomerular and lung basement membrane
Pemphigus vulgaris	Desmosomes between prickle cells in epidermis
Pemphigoid	Basement membrane
Phacogenic uveitis	Lens

Sympathetic ophthalmia	Uvea
Autoimmune haemolytic anaemia	Erythrocytes
Idiopathic thrombocytopenic purpura	Platelets
Primary biliary cirrhosis	Mitochondria (pyruvate dehydrogenase)
Active chronic hepatitis	Smooth muscle/nuclear lamins/nuclei
(HB_s-ve)	Cell surface lipoproteins
Ulcerative colitis	Colon 'lipopolysaccharide'
	Colon epithelial cell surface protein
Sjögren's syndrome	SS-A(Ro); SS-B(La)
	Ducts/mitochondria/nuclei/thyroid
	IgG
Rheumatoid arthritis	IgG
	Collagen
Discoid lupus erythematosus	Nuclear/IgG
Scleroderma	Nuclear/IgG/centromere
	Nuclear/IgG/Sc-70
Dermatomyositis	Nuclear/IgG/Jo-1
Mixed connective tissue disease	Extractable nuclear materials
Systemic lupus erythematosus	DNA
	Sm ribonucleoprotein
	Nucleoprotein
	Cytoplasmic sol.Ag.
	Array of other Ag incl. formed
	elements of blood/clotting factors/IgG
	Cardiolipin
Wegener's granulomatosis	Neutrophil cytoplasm (ANLA)

The concentration of antigen used in methods of the invention affects the affinity of the resulting human monoclonal antibodies. Generally, the binding strength of an antibody receptor for an antigen will be determined by the usual equilibrium constant of the reaction of antigen and surface antibody. When an appropriate number of antigen molecules are bound to the antibody receptors on the peripheral blood lymphocyte surface, the lymphocyte can be stimulated to form a lymphoblast. When only small amounts of antigen are present, only those lymphocytes with high-affinity antibody receptors will be able to bind sufficient antigen for stimulation to occur. Their daughter cells will, of course, also produce high-affinity antibody. Consideration of the antigen-antibody equilibrium equation shows that, as the concentration of antigen is increased, even antibodies with relatively low affinity will bind more antigen. Therefore, with high doses of antigen, lymphocytes with lower-affinity antibody receptors will also be stimulated. Thus, low amounts of antigen are generally favored to produce high-affinity antibody producing clones. High antigen concentrations give rise to an antiserum with low to moderate affinity. The preferred concentration of antigen used in the methods of the invention range from about 50 ng antigen per ml of culture (containing about $3x10^6$ lymphocytes) to no greater than 10 micrograms of antigen per ml of culture. Particularly preferred concentration ranges are between about 50ng to about 2.5μg per ml of culture.

D. Uses of human monoclonal antibodies

The human monoclonal antibodies produced by methods of the invention are particularly preferred in human therapeutic methods since they will not initiate an immune response when administered into a human subject.

The potential targets to which the present human monoclonal antibodies can be directed will be virtually unlimited, constrained only by the ability of a particular antigen to generate an immune response (i.e. memory B-lymphocytes) in a first donor, and by its availability in the present method to initiate a secondary immune response in the PBL's. Potential human targets can include: lymphocyte and bone marrow cell determinants such as CD4, CD8, CD33 and CD34; bacterial toxins; drugs of abuse; intoxicants; pharmaceuticals; tumor cell markers; cell development markers; viral and parasitic antigens; immunoglobulin epitopes and idiotypes; T-cell antigen receptor epitopes and idiotypes; neurological markers such as acetylcholine receptor, GABA receptor and myelin and cellular adhesion molecules such as ICAM-1 and LFA-1. See M. Isobe et al., "Specific Acceptance of Cardiac Allograft after Treatment with antibodies to ICAM-1 and LFA-1", Science, 255:1125-1127 (1992).

The human monoclonal antibodies produced by methods of the invention can be linked to a cytotoxic agent as an immunotoxin. Thus, immunotoxins of the present invention will have three key components, the targeting monoclonal antibody molecule, a bond

(preferably covalent) which holds the antibody and toxin together, and the cytotoxic agent.

Human monoclonal antibodies produced by the methods of the invention can also be used as blocking antibodies to protect cells with certain target marker antigens from the effect of other agents. Human monoclonal antibodies of the invention can also be used as radioimaging agents. Antibodies, or antibody fragments such as the Fab, Fab' and $F(ab')_2$ fragments can be labeled with radiometal isotopes such as Indium-111 and Technetium-99m, both of which are suitable for detection by external scintigraphy. These gamma-emitters are preferred because they are detectable with a gamma camera and have favorable half lives in vivo. Antibodies of the invention can be radio-labelled by any of the techniques known in the art. See Berger et al. U.S. Patent No. 5,024,829, the entire contents of which are incorporated herein by reference.

Human monoclonal antibodies of the invention can be coupled to a drug or therapeutic agent to be used in an in vivo.drug delivery system.

Examples

The following example illustrates production of human monoclonal antibody directed against human IgE.

1. Identification of Suitable Donors

An exemplary EIA for detecting anti-IgE serum in a donor was performed by first coating 96-well,

flat-bottomed EIA plates (Corning Glass Works, Corning, NY Cat. # 25801) with IgE (10 µg/ml in 0.1 M carbonate buffer, pH 9.4). Preferred human IgE was obtained from culture supernatants of cell line U266B1 (ATCC # TIB 196). About 50 microliters of antigen solution were added per well and held overnight at about 4°C.

Next, each well was washed three times with a solution of phosphate-buffered saline (PBS) (pH 7.2) - 0.05% Tween 20 (Sigma Chem. Co., St. Louis, MO Cat. # P1379). After this, 100 microliters of 1% bovine serum albumin (BSA, Sigma Cat. # A-7030) - phosphate buffered saline was added to each well and held for 1 hour at about 37°C in a humidified incubator. The wells were washed with PBS - 0.05% Tween 20, as above.

About 50 microliters of donor serum dilutions (in triplicate) were added to the wells and held at about 37°C, as above for 2 hours. Dilutions were in PBS - 0.5% BSA. The wells were washed with PBS - 0.05% Tween 20, as above.

Next, about 50 microliters of anti-human IgG-alkaline phosphatase conjugate (Sigma Cat. # A-3150) was added to each well at a dilution in PBS of 1: 2,500. The wells were held at 37°C for 2 hours. Washing with PBS - 0.05% Tween 20 was then performed, as above.

To each well was added about 50 microliters of Sigma 104 phosphatase substrate (Sigma Cat. # 104-0) dissolved at 1 mg/ml in Sorensen's glycine buffer II supplemented with 0.01% $MgCl_2$ and 0.2% sodium

azide. The absorbance was read at 405 mm with a Biotek 2000 (Fisher Scientific Co., Medford, MA) microplate reader.

Results for this assay are shown in Figure 1. The letters (except BGG) represent human donors. Absorbance values at different dilutions are plotted on the Y-axis. TB and HB were patients that had an autoimmune condition and thus were expected to give positive results while BW and JWH were not. BGG is bovine gamma globulin as a negative control.

2. Screening of Active PBL's

An exemplary ELISPOT assay for detection and enumeration of IgE-specific peripheral blood lymphocytes includes first coating 6 cm Petri plates (Falcon 1007) with 2 ml of human IgE solution (Scripps Laboratories, San Diego, CA, Cat. # 10224) at a concentration of 10 µg/ml of sterile PBS. The plates were incubated overnight at 4°C. The plates were washed three times with PBS-0.05% Tween 20.

Next, excess binding sites on the plates were blocked by incubating the plates for about 6 hrs at about 37°C with 3 ml/plate of 2% CPSR-3 in sterile PBS. Plates were washed three times with PBS-0.05% Tween 20, as above.

Next, two or four mls of PBL's were added to each plate. PBL's were contained in RPMI-1640 (Mediatech Cat. # 60-002-PE) supplemented with 10% CPSR-3, 2mM L-glutamine (Sigma, Cat. # G-7513), 100 µg/ml gentamycin (Sigma Cat. # G-1397), 2mM sodium pyruvate

(Sigma, Cat. # S-8636), 2mM non-essential amino acids (Mediatech Cat. #25-025-LI), and $1-5X10^{-5}$ molar 2-mercaptoethanol (Sigma Cat. # M-6250). Plates were incubated overnight at 37°C in a 5% CO_2 humidified incubator. The plates were then washed three times with PBS-0.05% Tween 20, as above.

About 3 ml of ice-cold 10 mM EDTA was then added to each plate. The plates were held at 4°C for about ten minutes and then washed three times with PBS-0.05% Tween 20.

Next, to each plate was added 2.5 ml anti-human IgG-alkaline phosphatase (Sigma Cat. # A 3150) at a dilution of 1:250. The diluent was sterile PBS. The plates were held at room temperature for about six hours and then washed four times with PBS-0.05% Tween 20.

To each plate was then added 1.5 ml 0.5% agarose (FMC Corp., Rockland, ME Cat. # 50002) in PBS containing BCIP/NBT (Stratagene, La Jolla, CA, Cat. Pico blue #200372). The mixtures were allowed to cool/react for 24 hours. At the end of this time, plaques were enumerated using a reading/magnifier glass.

Individuals with greater than 50 plaques/10^6 PBL's plated are considered to have an elevated response sufficient for use in the immunization/fusion steps.

3. Immunization and Activation of PBL's

Peripheral blood lymphocytes were obtained from venous blood of the donor into heparinized vacutainer-tubes (Becton Dickinson Co. Cat. # 6482). About 15 tubes of blood were drawn. The blood was pooled and processed with Accuspin-tubes (Sigma Cat. # A7054) according to the manufacturer's instructions. Centrifugation was done at 1000 x g (2300 RPM) for 10 minutes at 25°C.

The PBL layer (buffy coat) was removed and cells further concentrated by centrifugation at 250 x g. Cells were washed twice in PBS and collected by centrifugation, as above.

Splenocytes were obtained from splenectomy patients within 6 hours after surgery. A single cell suspension was prepared by forcing spleen tissue fragments through a no. 50 mesh wire screen. Cells were then collected by centrifugation at 250 x g for ten minutes and red blood cells removed by ammonium chloride lysis. The remaining cells were washed, resuspended in a medium consisting of 40% RPMI, 50% FCS and 10% DMSO at a concentration of 100 to 300 x 10^6 cells/ml, frozen in 1.5 ml aliquots and stored in liquid nitrogen.

Frozen splenocytes were thawed at 37°C and washed twice with 15 ml RPMI. Centrifugation was carried out for 10 minutes at 250 x g. After the second wash, approximately equal numbers of splenocytes and PBL's were mixed and distributed into the inner wells of a 24-well tissue culture dish at 3 x 10^6 cells/ml at a

volume of 2 ml/well. The culture media used is that referred to in the fusion protocol, below, but without the selective HAT components. The presence of 2-mercaptoethanol in the media enhances the capacity of the B-lymphocytes to incorporate the sulfur-containing amino acids they require for immunoglobulin synthesis. H. Ohmari and I. Yamamoto, J. Exp. Med., 155:1277-1290 (1982).

Immunizing antigen (human IgE) was immediately added to the cultures in the tissue culture dish at the concentrations described and the cultures then incubated at 37°C for 3-5 days prior to fusion.

Human IgE immunized allogeneic spleen x peripheral blood lymphocyte cultures were then harvested from 24 well plates by gentle scraping and pipetting using a 5 ml pipette. Cells from the same plate were pooled into 50 ml conical centrifuge tubes and centrifuged for 5 min. at 1500 rpm. These cells were then washed twice, resuspended in fusion media and counted. Viabilities were determined by Trypan Blue dye exclusion.

4. Preparation of Fusion Product

A solution of 42% polyethylene glycol (PEG 1450) (American Type Culture Collection, Rockville, MD)/15% dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, Cat. #D-8779) was made by adding 4 grams of PEG to 1.4 ml DMSO and adjusting the final volume to 9.5 ml with RPMI 1640 (Mediatech, Washington, DC, Cat. #15-040-LV). The pH was adjusted to 7.2 by adding

several drops of Gey's C (sodium bicarbonate 0.66M). A 2.5% CPSR3 (Sigma, Cat. #C-0786)-IDMEM (Mediatech, Cat. #15-016-LV)/RPMI 1640 (50/50) solution was made for fusion media.

A 50x HAT (hypoxanthine, azaserine, thymidine) media supplement was prepared by adding 10 ml IDMEM to 50x HT media supplement (Sigma, Cat. # H-0137), then adding this to 50x Azaserine (Sigma, Cat. #A-1164). Final HAT media consisted of 50/50 IDMEM/RPMI 1640 [Final Media components: 25mM HEPES buffered, (Mediatech, Cat. # 60-002-PE), 2mM L-glutamine (Sigma, Cat. # G-7513), 100ug/ml gentamycin (Sigma, Cat. # G-1397), 2mM sodium pyruvate (Sigma, Cat. # S-8636), 2mM non-essential amino acids (Mediatech, Cat. # 25-025-LI), 10% CPSR3, 5×10^{-5} molar 2-mercaptoethanol (Sigma, Cat. # M-6250), 136 ug/ml hypoxanthine, 1 ug/ml azaserine, 3.88 ug/ml thymidine].

K6H6/B5 heteromyeloma cells in log phase (American Type Culture Collection # CRL 1823) were harvested by centrifuging for 5 min. at 1500 rpm. Cells were washed once with RPMI 1640 then resuspended in fusion media for counting. Viability was determined by Trypan Blue dye exclusion.

Cell counts were adjusted to provide a ratio of 2:1 to 4:1 splenocyte/PBL : K6H6/B5. Cells were pooled in a 50 ml conical centrifuge tube and centrifuged for 5 min. at 800 rpm. This cell suspension was resuspended in 20 ml fusion media and 0.5 ml was pulled off to use as a control in the subsequent fusion (no PEG). Cells were centrifuged

again for 5 min. at 800 rpm. Media was aspirated off the cell pellet and the pellet was gently tapped to resuspend the cells.

Over a period of 1 min., 1 ml PEG/DMSO was added to the cell pellet while rotating the 50 ml conical tube to gently stir the contents. The conical tube was rotated for an additional 2 minutes (clumping should be visible). Over a period of 1 minute, 1 ml of 2.5% media was added with rotation. Over 1 minute, an additional 1 ml of media was added with rotation. For the next 1 minute, 2 ml of media was added dropwise with rotation of the conical tube. Over a 2 minute period, 5 ml of media was added. Finally, 12 ml of 2.5% media was added dropwise with rotation. The cells were then centrifuged for 5 min. at 800 rpm. Media was decanted and the fusion products were resuspended in HAT media at a K6H6/B5 concentration of approximately 2.5×10^5 cells per ml for plating.

Fusion products were seeded onto a 96 well tissue culture plate (Corning, Corning Glass Works, Corning, NY, Cat. # 25860) at 200 microliters per well. Control cells were resuspended in 5 ml HAT media and seeded into 2 wells per primary fusion plate. Plates were incubated at 37°C in a 5%CO_2 incubator. After 4 days, each well received 1 drop of fresh media. Wells were then fed regularly about every 5 days by aspirating some media and adding 1 drop of fresh media. Clones were visible by inverted microscope observation on day 5.

5. Production, Screening and purification of human anti-human IgE monoclonal antibody

Wells that contained successful fusions were screened by observing those wells that are growth-positive using a phase contrast microscope. Hybridomas present in these growth-positive wells are screened for antibody production by ELIFA for reactivity on the immunizing antigen.

An exemplary ELIFA protocol includes assembling an ELIFA apparatus (Pierce Chemical Company, Rockford, IL, Cat. #77000) following the manufacturer's instructions using a Biodyne A membrane (Pierce, Cat. #77015) pre-wetted with distilled water. Residual water is removed by suction.

The membrane was then coated by addition to each well of 100 microliters of human IgE (Scripps Laboratories, San Diego, CA, Cat. # 10224) at 2.5 micrograms/ml 0.1 M carbonate buffer, pH 9.4. Next, excess binding sites were blocked by adding 200 microliters of PBS-2% BSA (Sigma Cat. # A7030, sterile filtered prior to use) to each well. After this, about 50 microliters of test supernatant or control solution were added to each well.

Human IgG antibody bound to the membrane was detected by adding to each well, 100 microliters of alkaline phosphatase conjugated goat anti-human IgG (Organon Teknika Corp., West Chester, PA, Cat. #59289) diluted 1:1000 in PBS-0.5% BSA . Each well was then washed twice with 200 microliters PBS-0.5% BSA.

The color was developed by adding 200 microliters Sigma 104 substrate (Sigma, Cat. #104-0) prepared as for the EIA of step 1. The wells were incubated for ten minutes at room temperature. Then, the color development is read in a Biotek 2000 microplate reader.

Cultures that are antigen specific and still positive after transfers were subcloned at a ratio of 1/3 cell/well to insure monoclonality. Cells can be cryopreserved in growth medium in 2 ml freeze vials at between 2 to 10 million cells/vials stored in liquid nitrogen.

Confirmation of antibody production was performed by using the EIA described previously for step 1.

For antibody purification, selected hybridomas were subcloned and expanded into 250-1000 ml spinner cultures in standard growth medium. All purification steps were performed at 4°C. Culture supernatants were mixed with 1 M potassium phosphate (pH 6) to a final concentration of about 0.1 M and applied to a column of protein G sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at a rate of 0.5 to 1.0 ml/min. After loading, the column was washed with 0.1 M potassium phosphate and eluted with 4X the column volume of 0.1 M glycine HCl, pH 2.8. Fractions were collected directly into a neutralizing buffer. Peak protein-containing fractions were pooled and concentrated by ultrafiltration on an Amicon microfiltrator (Beverly, MA). Purified IgG was stored in 40% glycerol at -20°C, in PBS at 4°C, or lyophilized and sealed in ampules under nitrogen for longer term storage.

Figures 2 and 3 represent the results of screening growth positive wells for production of human anti-human IgE monoclonal antibody (IgG). From 2880 wells seeded with fusion products, about 1500 (52%) demonstrated growth in HAT medium. Of these, fourty-one were screened and 29 (70%) show IgE binding levels above background (Figure 2: samples 1-24; Figure 3: samples 25-41). The negative controls are the media and supernatants used by Boerner et al., J. Immunol. 147: 86-95 (1991). The positive control for this screening is serum taken from one of the seropositive donors (BW: see Figure 1).

Table III, below, compares fusion products made by Boerner et al., infra, and those made by the present method.

TABLE III: Comparison of IgG-Secreting Fusion Products

Fusion	No. wells/ fusion	No. clones	No. antigen-specific clones	antigen-specific
Boerner et al.[a] (spleen x spleen)	-	678	4	0.58
Present Work				
(spleen x spleen)[b]	960	378	274	72.4
(spleen x PBL)[c]	960	184	95	51.6
(spleen x PBL)[d]	960	86	56	65.1

a. Human ferritin as antigen (see Boerner et al., Table IV - page 91).

b. Human IgE as antigen. Fusion procedures as per Boerner et al., except presence of 2-mercaptoethanol in culture media.

c. Human IgE as antigen; PBL donor JWH (see Fig. 1).

d. Human IgE as antigen; PBL donor BW (see Fig. 1).

This Table demonstrates the remarkable effect of 2-mercaptoethanol on improving production of spleen x spleen hybrids over that reported by Boerner et al. for similar cultures. The percentage of total clones that produced functional hybrids increased more than 2 orders of magnitude over Boerner et al.. Allogeneic spleen x PBL fusions also yielded at least 50% of total clones showing antigen-specific activity.

Equivalents

It should be understood that the foregoing description of the invention is intended merely to be illustrative thereof, that the illustrative embodiments are presented by way of example only, and that other modifications, embodiments, and equivalents may be apparent to those skilled in the art without departing from its spirit.

Having thus described the invention, what we desire to claim and secure by Letters Patent is:

CLAIMS

1. A method of making a human monoclonal antibody that is specific against a selected antigen, comprising

forming a mixture of:

(i) a preparation of human lymphocytes, the preparation enriched for memory B lymphocytes and obtained from a donor that is seropositive for the selected antigen;

(ii) a preparation of human lymphocytes from a second donor that is allogeneic with respect to the first donor; and

(iii) the selected antigen, the preparations and the antigen present in sufficient quantities and subjected to conditions whereby a mixed lymphocyte response is generated between lymphocytes of said preparations, characterized by the production of secondary lymphoblast progeny of the memory B lymphocytes,

fusing at least some of the secondary lymphoblast progeny with heterohybrid fusing partners, and

isolating a fused cellular product that secretes the antibody with binding specificity for the selected antigen.

2. A method as claimed in claim 1 wherein a fused product that secretes an antibody with binding specificity for human self-antigen is selected.

3. A method as claimed in claim 2 wherein the fused product secretes an antibody with binding specificity for human immunoglobulin selected from the group consisting of human IgA and human IgE.

4. A method as claimed in claim 2 wherein the fused product secretes an antibody with binding specificity for human CD antigens.

5. A method as claimed in claim 2 wherein the fused product secretes an antibody with binding specificity for a cancer antigen.

6. A method as claimed in claim 2 wherein the memory B-lymphocytes are obtained from a donor with an autoimmune disease and wherein the antigen is a self-antigen involved in said autoimmune disease said self-antigen selected from the group consisting of self-antigens of Table II.

7. A method as claimed in claim 6 wherein the fused product secretes an antibody with binding specificity for human immunoglobulin.

8. A method as claimed in claim 7, wherein the human immunoglobulin is selected from the group consisting of human IgA and human IgE.

9. A method as claimed in claim 1, wherein the seropositive donor is seropositive for a foreign antigen.

10. A method as claimed in claim 9 wherein the foreign antigen is an epitope of an infectious agent and wherein the memory B-lymphocytes are obtained from a donor exposed to said infectious agent.

11. A method as claimed in claim 9, wherein the foreign antigen is an environmental toxin and wherein the memory B-lymphocytes are obtained from a donor exposed to said environmental toxin.

12. A method as claimed in claim 1 wherein the preparation of lymphocytes from the second donor includes splenocytes.

13. A method as claimed in claim 1 wherein the memory B-lymphocytes are obtained from a nonhuman mammal having a human immune system.

14. A method as claimed in claim 13, further comprising:

immunizing a SCID mouse with the selected antigen, and

collecting the memory B cells from the SCID mouse.

15. A method as claimed in claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 wherein the preparation of human lymphocytes enriched for memory B-lymphocytes is a preparation of peripheral blood lymphocytes obtained from blood or lymph nodes of the first donor.

16. A method as claimed in claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 wherein a fused product that secretes an antibody having a high affinity for the selected antigen is obtained.

17. A method of making a human monoclonal antibody that is specific for a selected antigen, comprising:

initiating secondary lymphoblast proliferation of memory B-lymphocytes by employing conditions of a mixed lymphocyte response, re-exposing said memory B-lymphocytes to said selected antigen and

immortalizing a secondary lymphoblast to form a monoclonal antibody secreting cell line that produces said human monoclonal antibody with a high affinity for the selected antigen.

18. A method as claimed in claim 17, wherein said proliferation is initiated in a preparation of human peripheral blood lymphocytes.

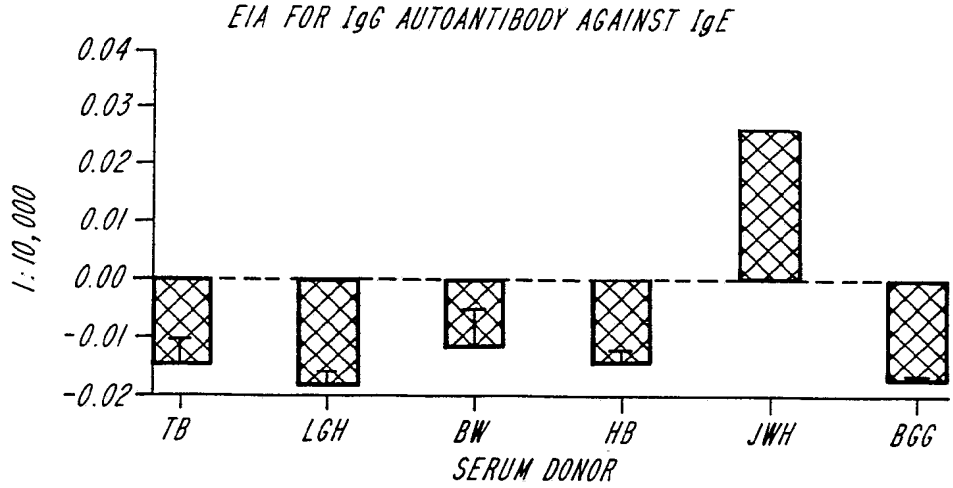

FIG. 1A

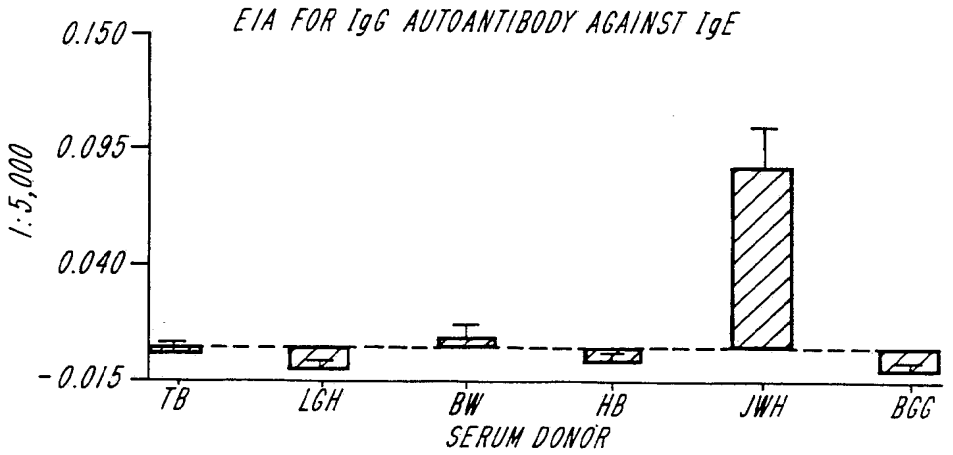

FIG. 1B

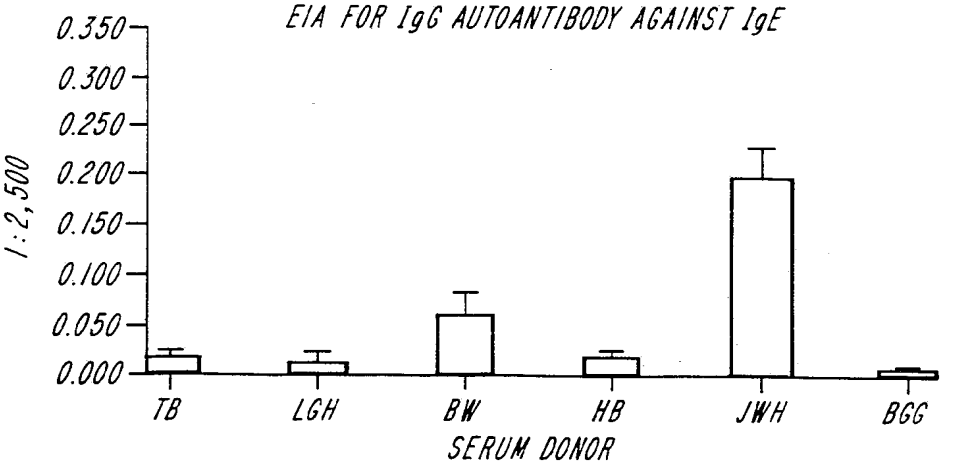

FIG. 1C

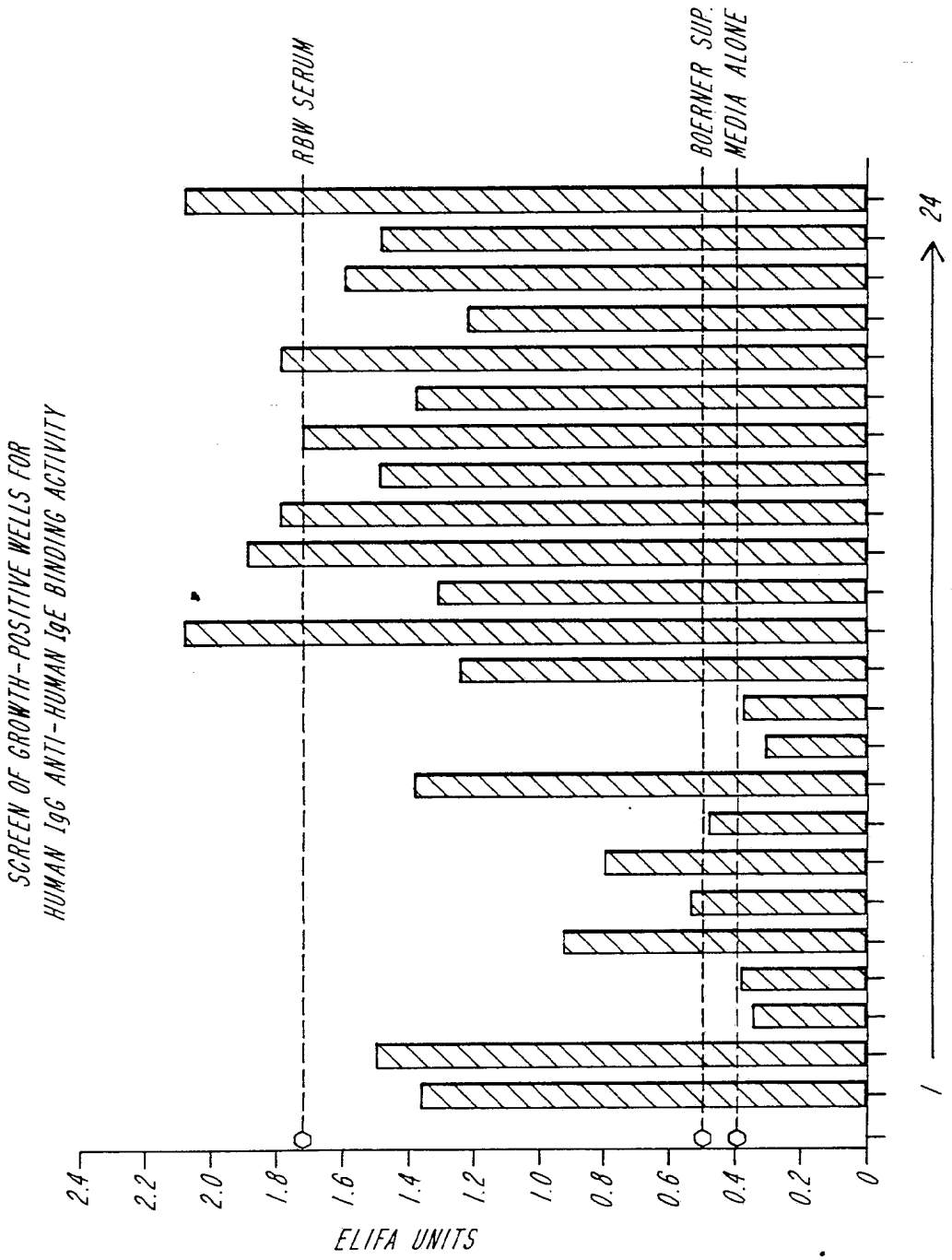
SCREEN OF GROWTH-POSITIVE WELLS FOR
HUMAN IgG ANTI-HUMAN IgE BINDING ACTIVITY
RBW SERUM
BOERNER SUP.
MEDIA ALONE
ELIFA UNITS
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
24
SAMPLES Nº 1 TO Nº 24

FIG. 2

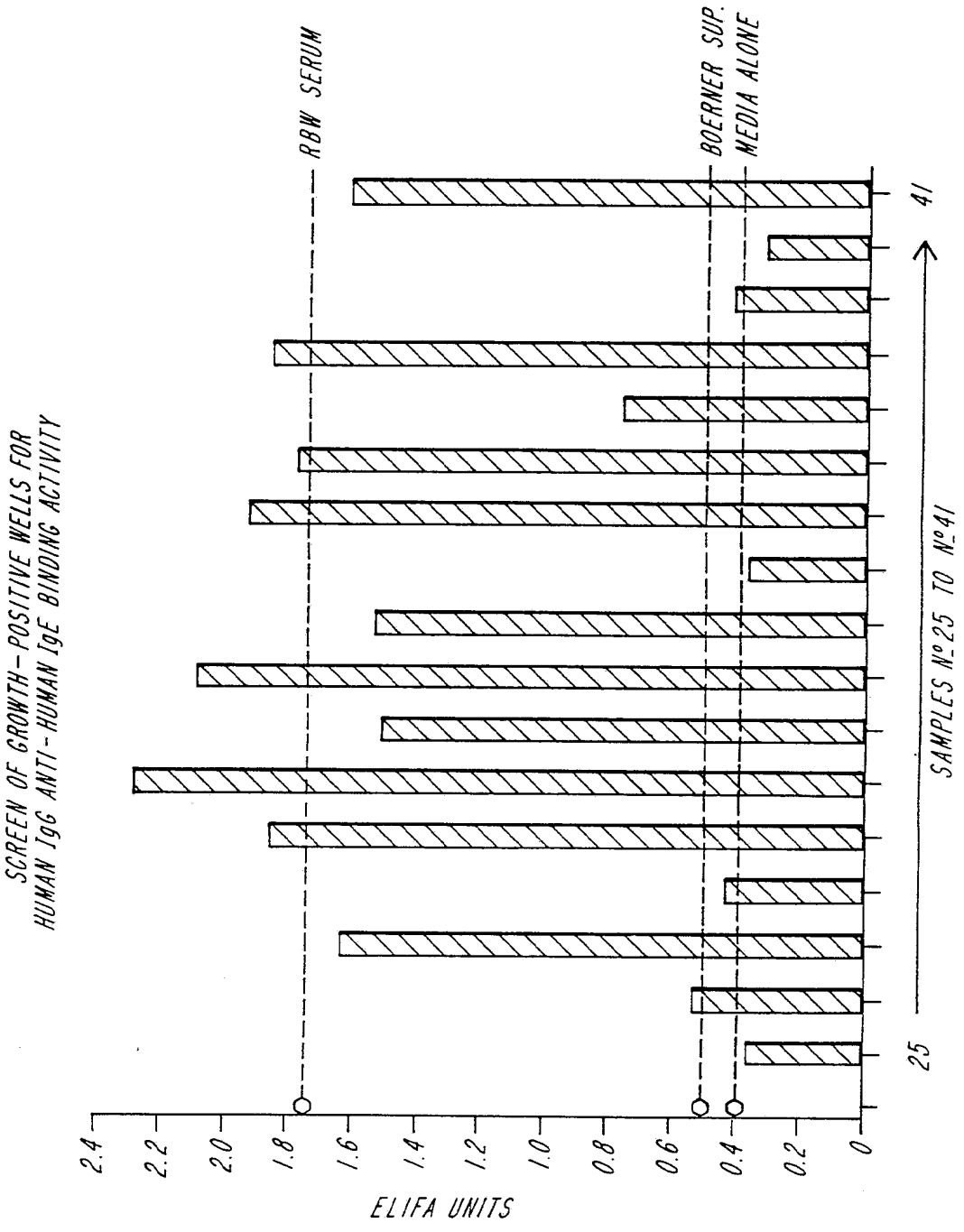
SCREEN OF GROWTH-POSITIVE WELLS FOR
HUMAN IgG ANTI-HUMAN IgE BINDING ACTIVITY
RBW SERUM
BOERNER SUP.
MEDIA ALONE
ELIFA UNITS
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
25
41
SAMPLES Nº25 TO Nº41

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/02479

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P21/08; C12N5/28; C12N5/20

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	US,A,4 716 111 (MICHAEL E. OSBAND ET AL.) 29 December 1987 see column 1, line 57 - column 3, line 29 ---	1
Y	PASCAL, ABSTRACT 90-0091458, BLANCHER A ET AL.:"HUMAN MONOCLONAL AUTOANTIBODIES PRODUCED BY HYBRISOMAS DERIVED FROM LYMPHOCYTES OF MULTIPLE SCLEROSIS PATIENTS.", & RESEARCH IN IMMUNOLOGY, 1989, 140 (7) 711-724,SEE ABSTRACT ---	1
A	WO,A,9 117 769 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 November 1991 ---	
A	EP,A,0 118 893 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 19 September 1984 -----	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 JULY 1993	26 07 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	REMPP G.L.E.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9302479
SA 71998

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 07/07/93

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